Part of #12

PATENT
Docket No. 20-4348P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicants: Eijiro WATANABE et al.

Serial No.: 08/992,914 Group: 1649

Filed: December 18, 1997 Examiner: O. Zaghmout

For: RAFFINOSE SYNTHASE GENES AND THEIR USE

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Eijiro WATANABE, citizen of Japan and residing in Fukui-cho 32-12-403, Takarazuka-shi, Hygo-ken, Japan, declare and say that:

1. I completed the doctor's course, with a major in agricultural chemistry, of the graduate school of Tokyo University and obtained a doctor's degree in agriculture at Tokyo University in March, 1991.

2. From April, 1991, I made further researches in the Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University, as a postdoctoral fellow (Japan Society for the Promotion of Science) for one year.

3. From April, 1992 to the present, I have been an employee of Sumitomo Chemical Company, Limited, the assignee of the above-identified application, and I have been engaged in research works for plant engineering using recombination and other gene manipulation, such as cloning of plant genes, preparation and evaluation of transgenic plants.

4. I am one of the inventors of the above-identified application and am familiar with the subject matter thereof.

5. I have read the Office Action mailed September 15, 1998 and the references cited, and am familiar with the subject matter thereof.

6. To demonstrate successful expression of raffinose synthase in transgenic plants, I have made the following experiments.

Experiments

Transformation of Tabacco with Broad Bean Raffinose Synthase Gene

The vector pBI121-RS having the broad bean raffinose synthase gene of the present invention in sense orientation, which is the same as obtained in Example 12 of the present specification, was used for the transformation of tabacco SR1 (*Nicotiana tabacum*) by the Agrobacterium infection method.

Agrobacterium tumefaciens (strain C58C1, rifampicin resistant) that had been previously made into a competent state by calcium chloride treatment was transformed with the above plasmid pBI121-RS. Screening of transformants was carried out on LB medium containing 50 μg/ml rifampicin and 25 μg/ml kanamycin by utilizing the character of kanamycin resistance conferred by the kanamycin resistance gene (neomycin phosphotransferase, NPTII) of the introduced plasmid.

The transformant Agrobacterium (*Agrobacterium tumefaciens* strain C58C1, rifampicin resistant) thus obtained was cultivated in LB medium containing 50 μg/ml rifampicin and 25 μg/ml kanamycin at 28°C for a whole day and night, and the culture was used for the transformation of tabacco by the method described below.

The leaves of tabacco plants aseptically sown and grown were cut into disk pieces with a surgical knife and infected with Agrobacterium (the culture obtained above) by the ordinary method as described by S.B. Gelvin, R.A. Schilperoort and D.P.S. Verma in *Plant Molecular Biology/Manual*,

published from Kluwer Academic Publishers in 1988. The disk pieces of the infected leaves were cultivated on MS-NB agar medium for 4 days. On the 4th day, the disk pieces were transferred to MS-NB agar medium containing 500 μg/ml cefotaxim for the elimination of Agrobacterium. On the 11th day, the disk pieces were transferred to MS-NB agar medium containing 500 μg/ml cefotaxim and 100 μg/ml kanamycin for starting the screening of transformant tabacco cells. After about 4 weeks, green differentiated seedlings were separated by cutting off, and transplanted into hormone-free MS agar medium containing 300 μg/ml cefotaxim and 50 μg/ml kanamycin. While further subculturing, screening of transformants was carried out by utilizing the character of kanamycin resistance to give tabacco plants each having the introduced gene of the present invention. The tabacco plants were allowed to cause rooting. The rooting plants were transferred to vermiculite : peat moss = 1 :1, and conditioned at 21° to 22 °C in a cycle of day/night = 12 hours : 12 hours. With the progress of plant body growth, the plants were suitably grown with cultivation soil. From the leaves of the regenerated plants, genomic DNA was extracted, and the gene insertion into the plant genome was confirmed by PCR using four primers as shown in the following list.

(List)

35S 30mer

TTCCAGTATGGACGATTCAAGGCTTGCTTC

NOS 25mer

ATGTATAATTGCGGGACTCTAATCA

RS-F 30mer

AAGAGTGTATCTGAATTTTCACGCGCGGTG

RS-RV 33mer

ACCTTCCCATACACCTTTTGGATGAACCTTCAA

Measurement of Raffinose Synthase Activity in Transformant Tabacco Plants

From the leaves of the transformant tabacco plants obtained above, a sample solution for the measurement of raffinose synthase activity was prepared as follows. To these tabacco leaves was added 100 mM Tris-HCl (pH7.4), 1 mM EDTA, 5 mM DTT, 1 mM PMSF (phenylemthylsulfonyl fluoride), 1 mM benzamide at a weight three times the weight of the leaves, and the mixture was ground with a mortar on ice. The resulting suspension was placed in a tube, followed by centrifugation at 10,000 x g for 5 minutes at 4°C. The supernatant was removed at a volume of 500 μl into a new tube. To 500 μl of this separated extract was added 1 ml of saturated ammonium sulfate (pH7.0), and the mixture was allowed to stand on ice for 30 minutes. The mixture was centrifuged at 10,000 x g for 5 minutes at 4°C. The resulting precipitate was dissolved in 50 μl of the same buffer as described above, and desalted with a Quant Probe column (Pharmacia) that had been previously equilibrated with the same buffer. The sample solution thus obtained was used for the measurement of raffinose synthase activity as described below. The amount of proteins was determined by the BioRad assay (BioRad).

The raffinose synthase activity was measured under the following conditions according to the description of L. Lehle and W. Tanner, *Eur. J. Biochem.*, 38, 103-110 (1973).

First, 2 μl of the sample solution was added to 18 μl of a reaction mixture that came to contain 100 mM Tris-HCl (pH7.4), 5 Mm DTT (dithio-

threitol), 0.01% BSA, 200 μM sucrose, 5 mM galactinol, 740 KBq/ml (31.7 μM) [^{14}C] sucrose at the final concentrations, and the mixture was kept at 37°C for 3 to 20 hours. After the reaction, 30 μl of ethanol was added to the reaction mixture, followed by stirring and centrifugation at 15,000 rpm for 5 minutes. Then, 5 μl of the supernatant was spotted on an HPTLC plate of cellulose for thin layer chromatography (Merck, 10 cm x 20 cm), and developed with n-butanol : pyridine : water : acetic acid = 60 : 40 : 30 : 3. After drying the developed plate, [^{14}C] raffinose produced was detected and determined with an imaging analyzer (Fuji Photographic Film, FUJIX Bio Imaging Analyzer BAS-2000II).

Results

The raffinose synthase activity was measured for four tabacco plants having the introduced plasmid pBI121-RS. The results are shown in the table below. The raffinose synthase activity was also measured for ~~one~~ six E.W. 03.Feb.1999 tabacco ~~plant~~ plants having no such introduced gene as a control (background), and E.W. 03Feb.1999 it was found to be 0.025 pmol.raffinose/μg.protein.hour (average of six plants).

TABLE

Sample	Raffinose synthase activity (pmol.raffinose/μg.protein.hour)
SR1(pBI121-RS) no.1	0.145
SR1(pBI121-RS) no.3	0.092
SR1(pBI121-RS) no.7	0.042
SR1(pBI121-RS) no.5	0.088

Discussion

As can be seen from the table above, all the samples each having the broad bean raffinose synthase gene of the present invention in sense orientation exhibited very higher raffinose synthase activity as compared with the control having no such gene. This indicates that tabacco plants may have highly improved raffinose synthase activity by introduction of the raffinose synthase gene of the present invention in sense orientation into these plants.

Thus, it is clearly demonstrated that the raffinose synthase gene of the present invention can be successfully expressed in the transgenic tabacco plants.

7. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

This 4th day of February, 1999

E. Watanabe

Eijiro WATANABE